[APPLICATION NOTE]

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

SIMULTANEOUS QUANTITATIVE DETERMINATION OF ETHYL GLUCURONIDE AND ETHYL SULPHATE IN HUMAN URINE USING UPLC®/MS/MS

Russell Watts[1], Jennifer Button[2], David W Holt[2], Michelle Wood[1]. [1]Waters Corporation, MS Technologies Centre, Manchester, UK. [2]Analytical Unit, St George's – University of London, London, UK.

OBJECTIVE

To develop and validate a simple and rapid UPLC/MS/MS method for the simultaneous quantitative determination of ethyl glucuronide (EtG) and ethyl sulphate (EtS) in human urine.

INTRODUCTION

- EtG and EtS are non-volatile, water soluble, direct metabolites of ethanol.
- Both EtG and EtS are formed shortly after ethanol consumption and can be detected much longer than ethanol itself (up to 80 hours for EtG, 30 hours for EtS)[1,2].
- EtG has been shown to be susceptible to post sampling synthesis[3] and also bacterial degradation[4,5] which may lead to false positive or negative results respectively. These effects were not seen with EtS, making EtS a more reliable marker.
- EtG and EtS are formed by different metabolic pathways and therefore simultaneous determination has been found to increase sensitivity and reliability in detecting recent ethanol consumption[6].
- EtG testing is now widely used in alcohol withdrawal programmes (to monitor abstinence), within workplace settings and for forensic cases such as post-mortems and drug-facilitated crimes.

Figure 1. System configuration-Waters ACQUITY® TQD

MATERIALS

Specimens

Validation was performed using human urine samples obtained from the Analytical Unit, St George's - University of London (London, UK) and Wythenshawe Hospital (Manchester, UK). All samples (Sodium fluoride preserved) were stored at -20 °C until analysis. Synthetic blank urine (Surine®, DYNA-TEK industries, USA) was used as the control material to prepare all the calibrators.

Internal standards

Deuterated analogues EtG-D5 and EtS-D5 (Lipomed, Switzerland) were used as the internal standards (IS). A mixed stock solution was prepared in water at 20 and 5mg/L respectively.

EXPERIMENTAL

Sample preparation

A simple urine dilution (1:20) was undertaken after centrifugation at 12000rpm (~11000xg) for 10 minutes.

This dilution also incorporated the addition of the IS. Briefly, IS (10µL) and 0.1% formic acid (940µL) were added to the human urine samples (50µL) before finally vortex mixing for 30 seconds.

LC Conditions

LC System:	Waters® ACQUITY UPLC
Column:	ACQUITY UPLC HSS C_{18} Column 2.1 x 150 mm, 1.8 µm
Column Temp:	50 °C
Flow Rate:	400 µL/min
Mobile Phase A:	Water containing 0.05% formic acid
Mobile Phase B:	Acetonitrile
Gradient:	1-100% B over 2.5 min
Injection Vol:	10µL
Strong Wash Solvent:	Mobile phase B (800µL)
Weak Wash Solvent:	Mobile phase A (2400µL)

MS Conditions

MS System:	Waters® TQ Detector mass spectrometer
Ionization Mode:	ESI Negative
Capillary Voltage:	2.5 kV
Acquisition mode:	Multiple reaction monitoring (MRM)
Data processing:	MassLynx® v4.1 with TargetLynx™

Compound	Precursor Ion (m/z)	Product Ion (m/z)
EtG	**221**	**85**
	221	75
EtS	**125**	**97**
	125	125
EtG-D5	**226**	**85**
EtS-D5	**130**	**98**

Table 1. MRM conditions used for EtG, EtS and internal standards. Bold transitions used as the quantifier ion.

RESULTS AND DISCUSSION

The MRM conditions used for the measurement of EtG, EtS and their respective internal standards are summarised in Table 1. A calibration curve (0.25–100mg/L for EtG, 0.05-20mg/L for EtS) was prepared by adding EtG and EtS to synthetic blank urine. Calibrators and quality controls (QC) were diluted by the same procedure as previously described for the samples.

Figure 2 shows the MRM chromatograms obtained from a 10µL injection of a 0.5mg/L urine calibrator. The quantifier/qualifier ion ratios for both compounds were monitored for all calibrators, QC's and samples and were found to be within ±20% of the target ion ratios.

Quantitation was performed by the integration of the area under the peak of the specific MRM chromatogram. Figure 3 shows a typical standard curve for EtG and EtS in urine. Calibrators were plotted using 1/x weighting and found to be linear for both compounds, over the investigated range (coefficient of determination r2 = >0.996).

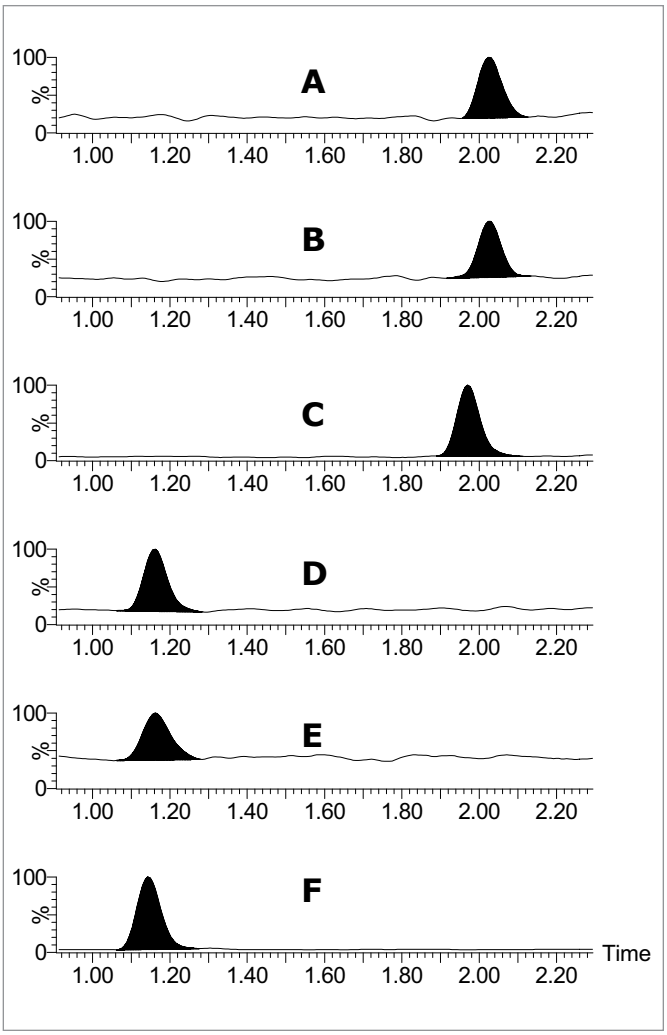

Figure 2. MRM chromatograms obtained from a 10µL injection of a urine calibrator at the cut-off level (0.5 and 0.1mg/L EtG and EtS respectively) for EtG quantifier ion (A), qualifier ion (B), EtG-D5 (C) and EtS quantifier ion (D), qualifier ion (E), EtS-D5 (F).

Limits of detection were 0.2 and 0.04mg/L for EtG and EtS respectively, which is below the cut-offs applied for this analysis i.e, 0.5 and 0.1mg/L respectively.

Intra-assay precision and accuracy were assessed by adding the EtG and EtS to blank patient urine (n=5) at four QC concentrations (0.75, 2.5, 7.5 and 50mg/L for EtG and 0.15, 0.5, 1.5 and 10mg/L for EtS). Inter-day precision was assessed by analysing the QC samples in duplicate on five different days. Intra and inter-assay precision and accuracy was found to be good, with precision CV's <10% and accuracy between 97-112%, as shown in Table 2.

The stability of prepared samples and standards was assessed over 24 hours. A prepared calibrator (2500/500mg/L, EtG/EtS) was stored at 5 °C in the dark in the ACQUITY sample manager with an injection performed every hour. No significant changes in absolute peak area were found for either compound over the investigated time period.

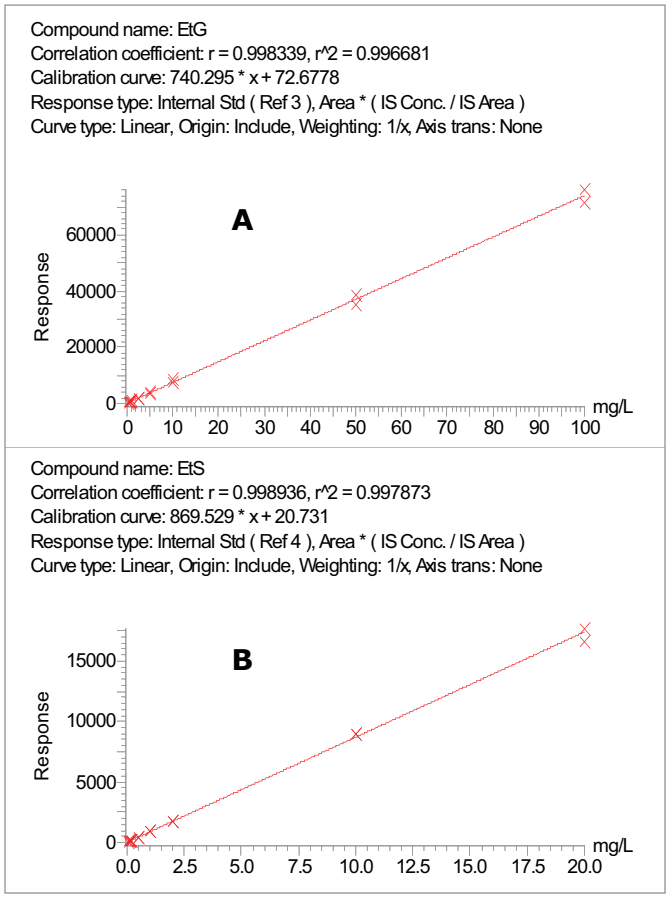

Figure 3. Typical calibration curves obtained for EtG (A) and EtS (B).

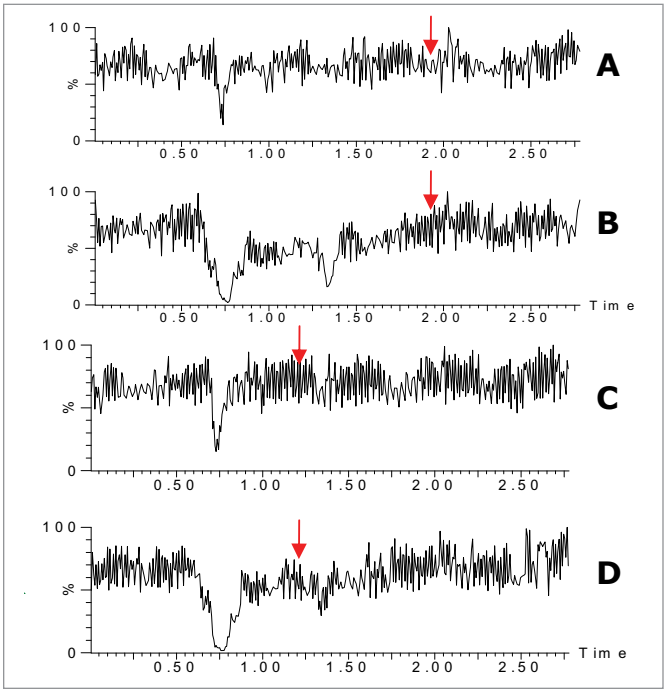

Figure 4. Chromatograms showing the post-column infusion of EtG (A+B) and EtS (C+D) at 1.0 and 0.2mg/L respectively, during the injection of solvent blank (A+C) and a prepared urine blank (B+D). Red arrows show the elution position of both compounds.

Matrix effects were assessed in 2 ways, firstly by spiking blank prepared patient samples (n = 6) with both compounds and comparing the absolute peak areas against the equivalent concentration of standard solution in solvent. The average matrix effects were found to be acceptable (-16% for EtG and -7% for EtS). Secondly, a post-column infusion of both compounds was performed during the injection of a solvent blank and prepared urine. Minimal matrix effects were observed with a simple urine dilution. An example shown in Figure 4.

Compound	QC Level (mg/L)	Accuracy (%) (n=5)	Intra-day Precision (CV %)(n=5)	Inter-day Precision (CV %)(n=10)
EtG	0.75	104.6	3.8	5.5
	2.5	103.3	5.8	8.0
	7.5	102.0	3.8	6.2
	50	111.8	8.3	9.3
EtS	0.15	103.2	1.7	5.7
	0.5	98.7	2.4	3.6
	1.5	97.3	2.0	4.1
	10	97.8	5.6	6.4

Table 2. Intra and inter-day precision and accuracy for EtG and EtS at 4 QC levels across the calibration range.

Sample No.	Ethanol (mg/dL)	EtG (mg/L) Immunoassay	EtG (mg/L) UPLC/MS/MS	EtS (mg/L) UPLC/MS/MS	Time post alleged DFSA incident (hrs)
1	174	171.7	184.4	42.8	4.5
2	126	1301.0	1751.7	294.0	8
3	<10	113.1	144.2	39.1	30
4	<10	Below cut-off	Below cut-off	0.2	3.5
5	<10	Below cut-off	Below cut-off	Below cut-off	55
6	<10	Below cut-off	Below cut-off	0.2	18
7	159	196.6	292.6	96.5	N/A
8	<10	1.766	254.7	37.5	8.5
9	<10	0.5	1.0	0.9	38
10	<10	0.5	1.6	0.7	12
11	186	272.7	401.9	131.3	12
12	<10	232.8	314.7	63.3	N/A
13	100	>2000	954.3	185.2	5
14	<10	6.1	10.5	1.9	1
15	<10	Below cut-off	Below cut-off	Below cut-off	16
16	55	54.7	75.6	19.9	6.5
17	197	950.0	1394.7	361.9	16
18	187	1332.0	1621.2	290.1	N/A
19	209	62.1	82.8	28.4	6.5
20	157	507.0	681.7	188.4	N/A
21	175	96.9	108.8	32.5	2
22	190	667.0	792.5	155.7	3.5
23	18	157.0	183.8	45.8	14
24	<10	8.8	13.2	5.7	14
25	<10	Below cut-off	Below cut-off	0.3	48
26	<10	Below cut-off	Below cut-off	0.4	48
27	161	543.0	692.6	273.6	3.5
28	47	812.0	1021.3	235.0	12.5
29	106	605.0	636.0	134.7	16
30	<10	Below cut-off	Below cut-off	Below cut-off	2
31	184	545.0	660.8	132.6	1
32	87	409.0	446.9	195.2	4
33	186	>2000.0	3385.3	838.9	6
34	116	63.2	83.3	17.8	5
35	<10	Below cut-off	Below cut-off	0.2	19.5
36	156	393.0	495.1	66.7	N/A
37	106	454.0	617.9	201.0	1
38	<10	ND	0.7	0.4	>48
39	<10	7.6	10.8	3.8	11

Table 3. EtG and EtS concentrations in analysed forensic case samples. Cut-offs of 0.5 and 0.1mg/L for EtG and EtS respectively, were applied to both the immunoassay and the UPLC/MS/MS method.

Forensic samples (n = 39) collected from alleged drug- facilitated sexual assault (DFSA) cases which had been previously analysed for EtG using the Microgenics DRI® EtG Enzyme Immunoassay[7], were subsequently analysed using the newly developed UPLC/MS/MS method. There is currently no immunoassay test available for EtS. EtG and EtS levels are highly influenced by water intake[8] therefore normalisation of EtG and EtS values to the creatinine concentration is recommended, but for the purpose of this comparison results were not normalised. Preliminary results showed that many samples contained EtG and EtS concentrations which were above the calibration range used. Therefore, all samples were re-prepared by dilution (1:100) with synthetic blank urine, as previously described and re-analysed. Table 3 shows the EtG and EtS results from the forensic case samples. The EtG results showed a good correlation (r^2=0.978) but also showed an analytical bias, as shown in Figure 5. The bias will be investigated in future work by the analysis of an independent reference material. EtG and EtS were detectable in samples collected up to approximately 40 hours after the alleged DFSA.

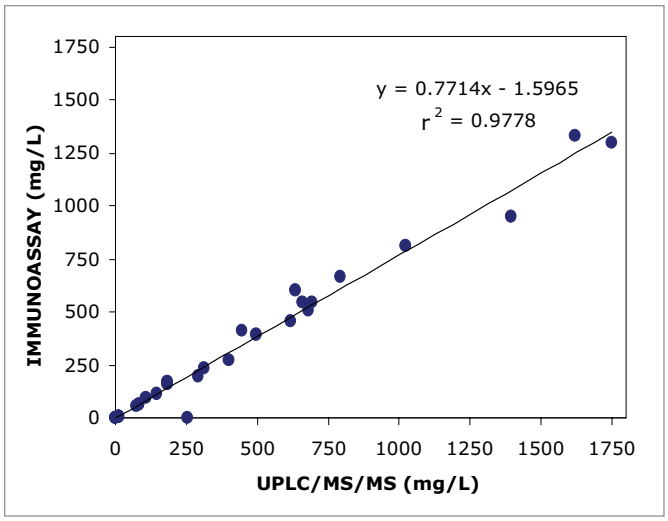

Figure 5. Analysis of EtG concentrations in forensic samples by immunoassay and UPLC/MS/MS. Results which were below the cut-off or >2000mg/L are not plotted.

CONCLUSION

EtG and EtS testing is becoming more widely used across the world within different settings such as alcohol withdrawal programs, clinical situations, forensic cases and the workplace to identify recent ethanol consumption or to verify abstinence.

The developed methodology has been shown to be accurate, precise and sensitive for the simultaneous quantitation of EtG and EtS and can provide rapid results in a single 4 minute chromatographic run.

The method has been successfully applied to the analysis of EtG in forensic samples with good correlation when compared to an established immunoassay. There is currently no immunoassay test available for EtS.

The speed and simplicity of the developed method make it the ideal solution for reliable, rapid, high-throughput EtG and EtS analysis.

ACKNOWLEDGEMENTS

Brian Keevil, Wythenshawe Hospital (Manchester, UK) for supplying blank patient urine samples for the validation process.

References

1. Wurst FM, Skipper GE, *Weinmann W. Addition.* (2003) 98(S2):51-61
2. Dresen S, Weinmann W, Wurst FM. J. Am. Soc. Mass Spectrom. 2004;15:1644-8
3. Helander A, Olsson I, Dahl H. Clin Chem 2007:53:1855-7
4. Baranowski S, Serr A, Thierauf A, Weinmann W, GroßePerdekamp M, Wurst FM, Halter CC. Int J Legal Med. 2008 Jun 24. [Epub ahead of print]
5. Helander A, Dahl H. Clin Chem 2005:51;1728-30
6. Wurst FM, Dresen S, Allen JP, Wiesbeck G, Graf M, Weinmann W. Addiction 2006:101(2):204-11
7. Button J, Mathers C, Elms R, Dhillon J, Holt D. Ann Toxicol Anal. 2008;20(S1):68
8. Goll M. J Anal Toxicol 2002;26:262-5

Austria and European Export (Central South Eastern Europe, CIS and Middle East) 43 1 877 18 07, **Australia** 61 2 9933 1777, **Belgium** 32 2 726 1000, **Brazil** 55 11 5094 3788, **Canada** 1 800 252 4752 x2205, **China** 86 10 8586 8899, **CIS/Russia** 7 095 336 7000, **Czech Republic** 420 2 617 1 1384, **Denmark** 45 46 59 8080, **Finland** 358 9 5659 6288, **France** 33 1 30 48 72 00, **Germany** 49 6196 400600, **Hong Kong** 852 29 64 1800, **Hungary** 36 1 350 5086, **India and India Subcontinent** 91 80 2837 1900, **Ireland** 353 1 448 1500, **Italy** 02 265 0983, **Japan** 81 3 3471 7191, **Korea** 82 2 820 2700, **Mexico** 52 55 5200 1860, **The Netherlands** 31 76 508 7200, **Norway** 47 6 384 60 50, **Poland** 48 22 833 4400 **Puerto Rico** 1 787 747 8445, **Singapore** 65 6273 1221, **Spain** 34 93 600 9300, **Sweden** 46 8 555 11 500, **Switzerland** 41 56 676 7000, **Taiwan** 886 2 2543 1898, **United Kingdom** 44 208 238 6100
All other countries: Waters Corporation U.S.A. 1 508 478 2000/1 800 252 4752

MassTRAK™
Forensic Solutions

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

August 2008 720002750EN KK-PDF

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com